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PRESERVATION OF THE PROPERTIES OF THE HOGCHOLERA VIRUS IN VIRULENT DEFIBRINATED BLOOD

TREATED WITH 0.3 PER CENT PHENOL AND BUFFERED AT 5.5 pH

TRANSLATION NO.

782

APRIL 1963

U.S. ARMY BIOLOGICAL LABORATORIES
FORT DETRICK, FREDERICK, MARYLAND

CCBL: FD3-3957 (T-68-7)
JPRS: R-3090-D

29 April 1963

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Translated for:

U. S. CHEMICAL CORPS BIOLOGICAL LABORATORIES
Ft. Detrick, Maryland

By:

U. S. DEPARTMENT OF COMMERCE
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PRESERVATION OF THE PROPERTIES OF THE HOGCHOLERA VIRUS IN VIRULENT DEFIBRINATED BLOOD TREATED WITH 0.3 PERCENT PHENOL AND BUFFERED AT 5.5 pH

- Rumania -

/Following is a translation of an article by I. Gheorghiu, T. Albu, and I. Nitoiu, of the Pasteur Institute, in the Rumanian-language periodical Lucrarile stiintifice (Scientific Proceedings), Vol. 4, 1960 (?), pages 193-201./

According to the data in the literature, blood collected from swine sick with hogcholera, when preserved without the addition of preservatives, keeps its infectiousness for a relatively short time after its collection.

HASKINS (7) estimated that it maintains the virulence less than 160 days, and it is fully destroyed after roughly 7 months (216 days).

By adding phenol in a final concentration of 0.75% (percent) and then preserving it at +5°C, ANDREEV (1) found that the virulence of the hogcholera blood disappears in 135 to 162 days. In the investigations made by ZUWERKALOW and SOLOMKIN (14) the adsorption of the hogcholera virus on Al-hydroxide was possible only at pH 5. In an article (3) published in the Bulletin of the International Epizootics Office it is shown that the virulence of hogcholera virus is maintained three times longer at pH 5 to 5.5 than at pH 7. SLAVIN (11) also asserts that the hogcholera virus (henceforth abbreviated: HCV) is well preserved in acid medium, while CHOPIN and work-associates (4) believe that pH 5.2 is the best for the maintenance of its virulence.

In acid medium and with the addition of buffer solution, this virus can be kept according to ZELJKO (13) for 14 days at 37°C, for 7 days without the buffer, while the phenol-treated virus never stands a 37°C temperature for 7 days. L. and P. BALOZET (2) found that glycerine has preservative action on this virus, because suspensions of hogcholera organs kept in glycerine remained virulent for 5 years. MIHAITA and STOICAN (9) found that the addition of sodium merthiolate at a final concentration of 1:1000 has not changed the virulence of

defibrinated hogcholera blood for 398 days, compared with the initial virulence. The HCV which is not mixed with antiseptic will cause hogcholera of lethal outcome even 398 days after its collection, in a dilution of 1:1,000,000.

KOVES, HEGYELI and GÖZSI (8) hold that HCV in a virulent serum vacuum-dried and preserved in cold will keep its properties for long time. With such a virus, good results were obtained in sero-virulization 15 years after preserving the material. SCHWARTZ and MATHEWS (12) state that a strain of lyophilized HCV kept without vacuum was still virulent after two years. DONATIEN and LESTOQUARD (5) believed that the HCV will not become modified at all after successive passages on sensitive hogs, and that a strain of a definite nature may be considered as a fixed virus. The same authors (6), but after seven years, established by the method of intradermal reaction (xeno-reaction) that a virus strain which is reproduced for ten years by passages on hogs, even though it keeps its pathogenicity, is much more inferior in antigenic power. According to ANDREEV (1), by the passage of the HCV on animals of this species, its attenuation is brought about, a phenomenon which also occurs when this virus is kept on other laboratory species of animals.

According to some investigators (DALE and ZINOBER, quoted by PLACIDI; 10), by the passage in series on hogs, the virulence of some HCV strains will increase for some specimens, while for others it will drop.

Personal Researches

Starting from the year 1955, a slight reduction was noticed in the efficiency of the anti-hogcholera products, compared with that found at the examinations in the preceding years.

Based upon some data in the literature which show that the HCV, kept by serial passage on hogs, may change its characteristics, we withheld a quantity of the virus for sero-virulization (virulent blood, phenolated 0.3% and buffered at 5.5, with a 2 M acetic buffer), whose viability in time we supposed to periodically check by inoculation of certain dilutions in hogs susceptible to hogcholera.

This would allow us on the one hand to suggest the prolongation of validity of this product, which now is only two months after the collection, while on the other hand, if the antigenic properties of the anti-hogcholera products prepared from the last passage of virus would prove to be weaker, then we could return to an older passage.

Working Method

From Series 149 HCV for serovirulization, prepared on 27 August 1955, from the Passage 32 of the Bucuresti production strain (the number 32 does not mean the real number of passages on hogs, since from its isolation and its use in production during the past 15 years it had over a hundred passages on hogs), the official expiration date of the series being 27 October 1955, we retained 1000 ml distributed in 20-50 ml vials.

Pathogenicity Test

At different time intervals within and after the official expiration date of validity, the presence of virus was tested in samples obtained and kept at 4-8°C by means of a biological test on hogs susceptible to hogcholera. The test hogs were of the York breed, quarantined for at least 10 days, weighing individually 35-40 kg at the moment of the checkup and being quartered in boxes and cages well disinfected in advance. In this experiment, from the mentioned samples the material was inoculated subcutaneously in 1:10,000 and 1:100,000 dilutions, each to 2-4 animals.

Of each group of experimental hogs, parallel with the inoculated hogs, two blank-control hogs were retained in another cage located near those in which the biologically tested animals were, but without the possibility of direct contact. This was for eliminating the hypothesis of an infection with an outside virus.

The blank-control hogs were kept until after the appearance or evolution of sickness in those inoculated with the sample material while they remained healthy; then they were given a control infection with a hard dose of virulent blood (1 ml virulent blood inoculated subcutaneously, i.e., roughly one million M.L.D. of the virus) simultaneously with the experimental hogs which did not show pathological symptoms, that is to say, around 12-14 days after the inoculation of the material.

The results of this biological test obtained in the period from 14 October 1955 to 8 August 1958 are given in Table 1.

Discussion

From the data presented in the table it is evident that at the time of the biotests no outside infection could have occurred since the blank-controls kept under similar conditions, yet without having a direct contact with the inoculated animals, remained healthy, or they became sick with hogcholera only after a control infection with 1 ml virulent blood. The behavior of the blank-control hogs after the control infection, when all had the acute type of experimental hogcholera

TABLE 1. Time Preservation of the Virulence of Hogcholera Virus Bucharest Strain, Passage No. 32, Intended for Sero

Order Number	Days After the Preparation	Denomination of Hog Groups According to Farms of Origin	Test of the Virus Samples and Result, Until 14 Days								
			Tested Hogs						Blank-Control Hogs		
			With 1:10,000 Dilution			With 1:100,000 Dilution					
			Number of Hogs	Dead or Victims of the Disease	Remained Healthy	Number of Hogs	Dead or Victims of the Disease	Remained Healthy	Number of Hogs	Died from Cholera or Other Causes	Remained Healthy
1	47	Ozun	4	4	--	2	2	--	2	--	2
2	112	Tg. Mures	4	4	--	2	2	--	2	--	2
3	197	Stupini	4	4	--	2	2	--	2	--	2
4	287	Stupini	4	4	--	2	2	--	2	--	2
5	331	Stupini	4	4	--	2	2	--	2	--	2
6	438	Stupini	4	4	--	2	2	--	2	--	2
7	504	Catalina	2	2	--	2	--	2	2	--	2
8	559	Ozun	4	3	1	2	2	--	2	--	2
9	621	Ozun	2	2	--	2	--	2	2	--	2
10	731	Risnov	2	1	1	2	--	2	2	--	2
11	778	Risnov	2	2	--	2	--	2	2	--	2
12	867	Tolvadia	2	2	--	2	--	2	2	--	2
13	924	Nohrich	2	2	--	2	1	1	2	--	2
14	997	Ozun	2	2	--	2	2	--	2	--	2
15	1050	Ozun	2	1	1	2	2	--	2	--	2
Total			44	41	3	30	19	11	30	--	30

(Treated with 0.3% Phenol and buffered at pH 5.5)
virulization (S= 169), prepared on 27 Aug 1958

The Result After Control Infection With 1 ml Virulent Blood						Minimum Ascertained Titre
Tested Hogs			Blank-Control Hogs			
Number of Hogs	Died of Cholera	Survived	Number of Hogs	Died of Cholera	Survived	
--	--	--	2	2	--	1/100,000
--	--	--	2	2	--	1/100,000
--	--	--	2	2	--	1/100,000
--	--	--	2	2	--	1/100,000
--	--	--	2	2	--	1/100,000
--	--	--	2	2	--	1/100,000
2	2	--	2	2	--	1/10,000
1	1	--	2	2	--	1/100,000
2	2	--	2	2	--	1/10,000
3	3	--	2	2	--	1/10,000
2	2	--	2	2	--	1/10,000
2	2	--	2	2	--	1/10,000
1	1	--	2	2	--	1/100,000
--	--	--	2	2	--	1/100,000
1	1	--	2	2	--	1/100,000
14	14	--	30	30	--	1/10,000 1/100,000

with a lethal outcome, is an evidence that the biotested hogs in the used lots have been susceptible to hogcholera. It should be mentioned however that the susceptibility of these hogs to infection has not been uniform and that in this respect there were differences both between the groups of used hogs and individually within the same lot. This matter is ascertained from the analysis of some data presented in the table in regard to the hogcholera production with material sampled at larger interval after the preparation.

Thus, while up to 438 days sampling inclusively, by inoculation with 1 ml of sample material either in the 1:10,000 or in the 1:100,000 dilution, all the hogs became sick with hogcholera, which was corroborated clinically and pathologically, when we made biological tests 504 days after the preparation on hogs of another source, only those became sick which were inoculated with the 1:10,000 dilution, while the hogs inoculated with the 1:100,000 dilution did not show any clinical symptom of hogcholera for 10 days, nevertheless after the control infection with 1 ml of virulent blood they again became sick with hogcholera. At the subsequent checkup made 559 days after the preparation on another group of hogs of another source as well as at the checkups made on hogs of the same source 997 and 1,050 days after the preparation, all hogs inoculated with the 1:100,000 dilution became sick with acute hogcholera of a lethal end.

It follows that, although the hogs of all groups used for the experiment had been susceptible to hogcholera, since they became sick after the control infection which was very strong, their susceptibility to the infection has not been the same, however, since there were groups in which the hogs had greater sensitiveness and groups in which the susceptibility of the animals was relative. This was in relation to their farm origin.

Apart from this, the finding is once again corroborated which was made in Rumania and in other countries in regard to the existence of an individual resistance that is more pronounced in some animals than in other of the same group.

To support this statement, we mention the cases No. 8. 13 and 15 of the Table from where it follows that each animal resisted against the sample infection at a 1:10,000 dilution, and this resistance was absolute since after the control infection they became sick with hogcholera; at the same time, the remaining hogs of the groups became sick with hogcholera after inoculation of the sample, even though they received the sample material in an 1:100,000 dilution.

Although, according to the researches which we made, in the virulent blood phenol-treated at 0.3% and buffered at pH 5.5 the HCV is preserved generally at 4-8°C until at least 1050 days -- which is the maximum term until we checked the samples -- nevertheless in this period

of time the virulence of the material has not remained identical with the initially established value. We make this statement on the basis that during the first 438 days of preservation we could produce hogcholera with the two dilutions of infectious material in hundred percent of the tested animals, independently of the origin of the hog groups (24 hogs inoculated with the 1:10,000 dilution and 12 hogs with the 1:100,000 dilution), while after this date only in a part of the biotested animals could the experimental hogcholera be produced.

Thus, between 559 and 1,050 days after the preparation, out of 18 hogs inoculated with the 1:10,000 dilution 15 animals became sick with hogcholera and three hogs resisted, which makes 16.6 percent, while with a dilution of 1:100,000 out of 18 animals tested only 7 got sick with hogcholera and 11 hogs resisted, which makes 61.1 percent. The hogs which resisted the inoculation with the above mentioned dilutions of the sample material have not been refractory to hogcholera, and none became immunized as a result of the sample inoculations with a viral dose that is considered to be below the infectious threshold, but they became sick with hogcholera after a control infection which was made approximately with a million M.D.L. of the virus.

Although the HCV, when kept in the defibrinated virulent blood that is treated with 0.3% phenol and buffered at pH 5.5 and maintained at 4-8°C, will stay pathogenic for hogs till after 1,050 days from the time of its preparation and can be recovered even in a dilution of 1:100,000, the fact that at larger interval after the preparation and at the used dilutions only a part of the animals will get sick indicates that with the time a portion of the viral elements will lose its viability and infective properties. As a matter of fact, no antigenic characteristics were preserved since the sample inoculation did not confer immunity, all the animals becoming sick with hogcholera as a result of the strong control infection.

* * *

In the first part of this work we showed that in the virulent blood, treated with 0.3% phenol and buffered at pH 5.5 and kept at 4-8°C, the HCV preserves its pathogenicity, and it can be recovered even 1,050 days after its preparation at least. It remains to be shown whether during the preservation its antigenic value also remains unchanged.

TEST OF THE ANTIGENIC PROPERTIES*

(*FOOTNOTE: At the comparative examination of the antigenic properties we had the cooperation of the Laboratory of Scientific Control through the collective Pascu L., Elefteresecu A., and Dan Fl., and of the Antihogcholera Product Section through the collective Surdan C., Popa M., Carp N. and Nica A.)

To solve this problem, at the term of 1,050 days of preservation, this virus was passed twice on hogs susceptible to hogcholera and quarantined in advance. This was done for the purpose of recovering and perhaps restabilizing it, and then, with the virulent material (defibrinated blood) of the hogs in the second passage, hogs were infected which were intended for antigen preparation for production. A part of the hogs was infected with 1 ml virulent blood of this passage (considered Passage No. 34) while another part was infected with the same amount of virulent blood from the same strain used in production and which during the 1,050 days was moreover subjected to 16 passages on hogs, i.e., Passage No. 50 for this work.

With the virulent material, blood and organs (spleen and lymphatic nodules) collected separately from these two categories of hogs for production, according to the rules in force, parallel series were prepared, i.e., from the blood, antihogcholera vaccine inactivated with glycerinated crystal violet, while from the suspensions of hogcholera organs, antihogcholera vaccines formol-treated and adsorbed.

With the vaccines prepared under the indicated conditions hogs of identical weight (35-40 kg), apparently healthy and susceptible to hogcholera were inoculated; the animals in one group were of the same age and of the same farm. The vaccination of the animals was made with variable vaccine doses in scale. For the formol-treated and adsorbed vaccine, the inoculation was subcutaneous with a vaccine dose from 2.5 to 20 ml on groups of 2-5 animals, while for the vaccine with crystal violet the dose was between 1.25 and 10 ml under the same conditions.

Twenty-one days after the vaccination, the vaccinated hogs, to ether with 6 hogs of the same group for blank-control which after the vaccination lived together with the vaccinated hogs, were subjected to control infection. They were subcutaneously inoculated with 1 ml virulent blood of the work strain of the Pasteur Institute, i.e., perhaps about one million M.L.D. of the virus.

The results obtained in this investigation are included in Table 2.

Discussion

Both the hogs vaccinated with the formol-treated and adsorbed vaccines and those vaccinated with the vaccine inactivated with crystalline violet, behaved normal from a clinical point of view during the whole time after the vaccination until the control infection.

After the control infection however, although the largest part of the vaccinated animals resisted and were taken off from the observation after 21 days as healthy animals, -- an evidence that they became immunized, -- nevertheless a portion of them became sick and died with the diagnosis of hogcholera within the 21 days of observation period.

TABLE 2. Comparative Result of the Immunizing Value of Adsorbed and/or Crystal-Violet Treated Antihogcholera Vaccine Prepared with Hogcholera Virus of Recent (50) and old (34) Passage

Dose ml	Antihogcholera Vaccine Adsorbed in Aluminum Hydroxide									
	From New Passage					From Old Passage				
	Number of Hogs and Result					Number of Hogs and Result				
	Vaccinated	Infected with Virus	Dead	Resisted		Vaccinated	Infected with Virus	Dead	Resisted	
				Number	%				Number	%
20	5	5	-	5	100	5	5	-	5	100
10	4	4	2	2	50	4	4	-	4	100
5	4	4	-	4	100	4	4	-	4	100
2.5	2	2	-	2	100	2	2	-	2	100
M[X]	-	1	1	0	0	-	2	2	0	0
Total	15	15	2	13	86.6	15	15	-	15	100

Table 2 continued

Table 2 (continued)

Dose ml	Antihogcholera Vaccine with Crystal Violet									
	From New Passage					From Old Passage				
	Number of Hogs and Result					Number of Hogs and Result				
	Vaccinated	Infected with Virus	Dead	Resisted		Vaccinated	Infected with Virus	Dead	Resisted	
				Number	%				Number	%
10	5	5	-	5	100	5	5	-	5	100
5	4	4	2	2	50	4	4	-	4	100
2.5	4	4	1	3	75	4	4	1	3	75
1.25	2	2	2	0	0	2	2	-	2	100
M[x]	-	2	2	0	0	-	1	1	0	0
Total	15	15	5	10	66.6	15	15	1	14	93.4

M[x] = blank controls

Thus, among the hogs vaccinated with the adsorbed vaccine which was prepared with antigen from the production passage (Passage 50), after the control infection two out of four hogs died from a dose of 10 ml vaccine, while hogs which were vaccinated with 20, 5 and even 2.5 ml vaccine remained alive and were then considered as having become immunized. Apart from the vaccine's dose, out of a total of 15 hogs vaccinated with vaccine doses between 2.5 to 20 ml, 13 hogs resisted the control infection, which makes 86.6 percent. On the other hand, the hogs vaccinated in equal number and with the same dose of vaccine that was similarly prepared with antigen from the older passage (Passage 32) after two consecutive passages on hogs (i.e., with Passage 34), all resisted the control infection, thus, aside from the dose, the immunity which was confered is considered 100 percent.

Since with these two formol-treated and adsorbed vaccine variants (prepared with new passage or with old passage of the virus) the animals which were vaccinated with smaller doses of the vaccine, i.e., with 5 and 2.5 ml were fully resistant in both cases, it could be supposed that in case of the two hogs of the variant of adsorbed vaccine prepared with new production passage (Passage 50) which died after the control infection no immunity was set up, due to not so much of the vaccine's quality but to their inability of becoming immunized for causes depending upon the organism. We should mention, however, that in case of this vaccine the immunity that develops is indeed in proportion with the inoculated antigenic volume, yet in the process of immunization the quality of the antigen is also of great importance. Relying upon this interpretation, we explain the non-immunization of these two hogs, which were vaccinated with 10 ml vaccine prepared from the antigen for which the new production passage was used, as being due to their reduced immunizability on the one hand, while on the other hand the immunizing value of this vaccine was also much reduced in comparison with the preparation made from the old passage of virus.

Even if as to the quality of the formol-treated and adsorbed vaccine prepared with the two kinds of antigen, even with the given interpretation, the comparative results do not allow to make indisputable conclusions in favor of the one made with the old passage, the immunogenic value of the vaccine with crystal violet prepared with virus from the old passage seems to be comparatively superior when compared with the vaccine prepared from the new passage. As it is found from the data obtained at the examination of the immunizing value of these two vaccines inactivated with crystal violet, to be seen in Table 2, at 10 ml dose, both variants have produced immunity in 100 percent of the vaccinated hogs.

However, with a 5 ml dose the vaccine prepared with virus from the new passage has immunized only 2 out of 4 hogs, i.e., 50 percent, while the vaccine from the old passage immunized 4 out of 4 hogs, or 100 percent of the animals. With a dose of 2.5 ml, in case of both vaccines, 3 out of 4 hogs were immunized, which makes 75 percent, while at a dose of 1.25 ml with the new passage none was immunized out of two hogs, which is zero percent, and with the old passage 2 out of 2 hogs were immunized, which makes 100 percent.

Aside from the vaccine's dose, the vaccine with crystal violet prepared from the current production passage of the virus (Passage 50) immunized only 10 out of the 15 vaccinated hogs, or 66.6 percent while the same vaccine prepared with virus of the old passage (Passage 34) immunized 14 out of the 15 vaccinated hogs, which makes 93.4 percent.

Summing up the results obtained at the two categories of the vaccines prepared with these two virus passages, and comparing the obtained immunity, indifferently from the used doses at vaccination,

it is found that 23 animals out of the 30 hogs (or 76.6 percent) which were vaccinated with 1.25 to 20 ml doses of the adsorbed and the crystal-violet-treated vaccines, prepared with the current production passage (Passage 50) of the virus, have resisted the control infection, in other words, they were immunized, while in the same number of animals, vaccinated with the same doses of the same vaccines, prepared however with the old virus passage (Passage 34) 29 animals, or 96.6 percent, had resistance against the control infection.

Conclusions

1. In virulent defibrinated blood treated with 0.3% phenol and buffered with acetic 2M buffer at pH 5.5 and preserved at 4-8°C, the hogcholera virus stays pathogenic until at least 1,050 days of preservation at which time hogcholera can be produced in susceptible hogs with 1 ml of a 1:100,000 dilution.

2. By a serial passage on susceptible hogs, at a given moment (after several hundred passages), although the virulence of the "Bucharest" Strain of HCV has remained unchanged, its antigenic property seems to be much diminished in comparison with a passage from the same strain which was kept as such for 1,050 days during which time the production strain was subjected to 16 passages on hogs in addition.

3. The antihogcholera vaccines (formol-treated tissue and adsorbed, and the one from virulent blood inactivated with crystal violet), prepared with virulent material from hogcholera-sick young sows that had been infected with a virus preserved outside the animal organism for 1,050 days, have produced an average immunity of 96.6 percent in the hogs susceptible to hogcholera and vaccinated with 1.25 to 20 ml (i.e., in 29 hogs out of 30 vaccinated animals).

The same vaccines, identically prepared with virulent material of young sows infected with the same virus strain which was additionally subjected to 16 passages on hogs during the 1,050 days, and used in the same dose and on the same number of animals, produced an average immunity of 76.6 percent (in 23 out of 30 vaccinated hogs).

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